In re Application of:
    Barbas III, *et al.*
Application No.: 09/500,700
Filed: February 9, 2000
Page 3

PATENT
ATTY. DOCKET NO.: SCRIP1160-4

C7 site, and Sp1C site) as listed on top of each panel. The protein concentration is labeled in nM beneath each lane, with a 2-fold serial dilution from left to right in each panel.

The paragraph at page 9 (line 7) has been amended to read as follows:

FIGURE 18 is a DNaseI footprint of MBP-C7-C7 and MBP-Sp1C-C7. A 220 bp radiolabeled fragment containing the binding site for MBP-C7-C7 (lanes 1-3) or MBP-Sp1C-C7 (lanes 4-6) was incubated with either 20 ug/ml of BSA (lanes 2 and 4) or the cognate binding protein (300 nM, lanes 3 and 6) in 1x Binding Buffer for 30 min. DNaseI footprinting was then performed using the SureTrack Footprinting Kit (Pharmacia) according to the manufacturer's instructions. Boxed region indicates the binding site sequence (SEQ ID NOS 71 and 72). Asterisk indicates the 3'-labeled strand. Lanes 1 and 4: G+A ladders.
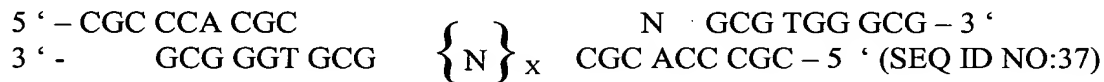
The paragraph at page 48 (line 30) through page 49 (line 32) has been amended to read as follows:

The Jun/Fos leucine zippers are described for illustrative purposes and preferentially form heterodimers and allow for the recognition of 12 to 72 base pairs. Henceforth, June/Fos refer to the leucine zipper domains to these proteins. Zinc finger Proteins are fused to Jun, and independently to Fos by methods commonly used in the art to link proteins. Following purification, the Zif-Jun and Zif-Fos constructs (SEQ ID NOS: 33, 34 and 35, 36 respectively), the proteins are mixed to spontaneously form a Zif-Jun/Zif-Fos heterodimer. Alternatively, coexpression of the genes encoding these proteins results in the formation of Zif-Jun/Zif-Fos heterodimers in *vivo*. Fusion of the heterodimer with an N-terminal nuclear localization signal allows for targeting of expression to the nucleus (Calderon, *et al, Cell*, 41:499, 1982). Activation domains may also be incorporated into one or each of the leucine zipper fusion constructs to produce activators of transcription (Sadowski, *et al., Gene*, 118:137, 1992). These dimeric constructs then allow for specific activation or repression of transcription. These heterodimeric Zif constructs are advantageous since they allow for recognition of palindromic sequence (if the fingers on both Jun and Fos recognize the same

In re Application of:
  Barbas III, *et al.*
Application No.: 09/500,700
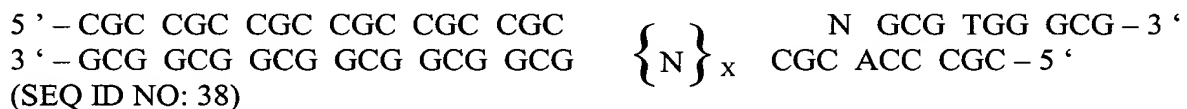Filed: February 9, 2000
Page 4

PATENT
ATTY. DOCKET NO.: SCRIP1160-4

DNA/RNA sequence) or extended asymmetric sequence (if the fingers on Jun and Fos recognize different DNA/RNA sequences). For example the palindromic sequence

$$5\,'-\text{CGC CCA CGC} \quad \left\{ N \right\}_x \quad N \quad \text{GCG TGG GCG}-3\,'$$
$$3\,'-\quad\quad\text{GCG GGT GCG} \quad\quad\quad \text{CGC ACC CGC}-5\,' \text{ (SEQ ID NO:37)}$$

is recognized by the Zif268-Fos/Zif268 Jun dimer (x is any number). The spacing between subsites is determined by the site of fusion of Zif with the Jun or Fos zipper domains and the length of the linker between the Zif and zipper domains. Subsite spacing is determined by a binding site selection method as is common to those skilled in the art (Thiese, et al., *Nucleic Acids Research*, 18:3203, 1990). Example of the recognition of an extended asymmetric sequence is shown by Zif(C7)$_6$-Jun/Zif-268-Fos dimer. This protein consists of 6 fingers of the C7 type (EXAMPLE 11) linked to Jun and three fingers of Zif268 linked to Fos, and recognizes the extended sequence:

$$5\,'-\text{CGC CGC CGC CGC CGC CGC} \quad \left\{ N \right\}_x \quad N \quad \text{GCG TGG GCG}-3\,'$$
$$3\,'-\text{GCG GCG GCG GCG GCG GCG} \quad\quad\quad\quad \text{CGC ACC CGC}-5\,'$$
(SEQ ID NO: 38)

The paragraph at page 50 (line 29) through page 51 (line 15) has been amended to read as follows:

Following mutagenesis and selection of variants of the Zif268 protein in which the finger 1 specificity or affinity is modified, proteins carrying multiple copies of the finger may be constructed using the TGEKP (SEQ ID NO:67) linker sequence by methods known in the art. For example, the C7 finger may be constructed according to the scheme:

MKLLEPYACP VESCDRRFSK SADLKRHIRI HTGEKP-

(YACPVESCDRRFSKSADLKRHIRIHTGEKP)$_{1-11}$, (SEQ ID NO:39) where the sequence of the last linker is subject to change since it is at the terminus and not involved in linking two fingers together. This protein binds the designed target sequence GCG-GCG-GCG in the oligonucleotide hairpin CCT-CGC-CGC-CGC-GGG-TTT-TCC-CGC-GCC-CCC GAG G (SEQ ID NO:40) with an affinity of 9nM, as compared to an affinity of 300 nM for an
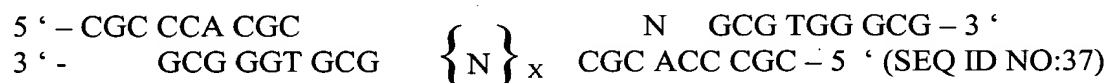
In re Application of:
    Barbas III, *et al.*
Application No.: 09/500,700
Filed: February 9, 2000
Page 5

PATENT
ATTY. DOCKET NO.: SCRIP1160-4

oligonucleotide encoding the GCG-TGG-GCG sequence (as determined by surface plasmon resonance studies). Fingers utilized need not be identical and may be mixed and matched to produce proteins which recognize a desired target sequence. These may also be utilized with leucine zippers (*e.g.*, Fos/Jun) or other heterodimers to produce proteins with extended sequence recognition.

The paragraph at page 81 (line 27) through page 82 (line 26) has been amended to read as follows:

The Jun/Fos leucine zippers preferentially form heterodimers and allow for the recognition of 12 to 72 base pairs. Henceforth, Jun/Fos refer to the leucine zipper domains of these proteins. Zinc finger proteins are fused to Jun, and independently to Fos by methods commonly used in the art to link proteins. Following purification, the Zif-Jun and Zif-Fos constructs (FIGURE 13 and 14, respectively), the proteins are mixed to spontaneously form a Zif-Jun/Zif-Fos heterodimer. Alternatively, coexpression of the genes encoding these proteins results in the formation of Zif-Jun/Zif-Fos heterodimers *in vivo*. Fusion with an N-terminal nuclear location signal allows for targeting of expression to the nucleus (Calderon, *et al., Cell*, 41:499, 1982). Activation domains may also be incorporated into one or each of the leucine zipper fusion constructs to produce activators of transcription (Sadowski, *et al., Gene*, 118:137, 1992). These dimeric constructs then allow for specific activation or repression of transcription. These heterodimeric Zif constructs are advantageous since they allow for recognition of palindromic sequences (if the fingers on both Jun and Fos recognize the same DNA/RNA sequence) or extended asymmetric sequences (if the fingers on Jun and Fos recognize different DNA/RNA sequences). For example the palindromic sequence

$$5\,'-CGC\ CCA\ CGC \qquad \left\{N\right\}_x \qquad \begin{matrix} N & GCG\ TGG\ GCG-3\,' \end{matrix}$$
$$3\,'- \qquad GCG\ GGT\ GCG \qquad \qquad CGC\ ACC\ CGC-5\,'\ (SEQ\ ID\ NO:37)$$

Is recognized by the Zif268-Fos/Zif268 Jun dimer (x is any number). The spacing between subsites is determind by the site of fusion of Zif with the Jun or Fos zipper domains and the length of the linker between the Zif and zipper domains. Subsite spacing is determined by a

In re Application of:
   Barbas III, *et al.*
Application No.: 09/500,700
Filed: February 9, 2000
Page 6

PATENT
ATTY. DOCKET NO.: SCRIP1160-4

binding site selection method as is common to those skilled in the art (Thiese, *et al., Nucleic Acids Research,* 18:3203, 1990). Example of the recognition of an extended asymmetric sequence is shown by Zif(C7) $_6$-Jun/Zif- 268-Fos dimer. This protein consists of 6 fingers of the C7 type (EXAMPLE 11) linked to Jun and three fingers of Zif268 linked to Fos, and recognizes the extended sequence:

$$5' - \text{CGC CGC CGC CGC CGC CGC} \quad \left\{ N \right\}_x \quad \text{N GCG TGG GCG} - 3'$$
$$3' - \text{GCG GCG GCG GCG GCG GCG} \quad \phantom{\left\{ N \right\}_x} \quad \text{CGC ACC CGC} - 5'$$

(SEQ ID NO: 38)

The paragraph at page 83 (line 5) has been amended to read as follows:

Following mutagenesis and selection of variants of the Zif268 protein in which the finger 1 specificity or affinity was modified (See EXAMPLE 7), proteins carrying multiple copies of the finger may be constructed using the TGEKP (SEQ ID NO:67) linker sequence by methods known in the art. For example, the C7 finger may be constructed according to the scheme:

MKLLEPYACP VESCDRRFSK SADLKRHIRIHTGEKP-
(YACPVESCDRRFSKSADLKRHIRIHTGEKP)$_{1-11}$, (SEQ ID NO:39), where the sequence of the last linker is subject to change since it is at the terminus and not involved in linking two fingers together. An example of a three finger C7 construction is shown in Figure 15. This protein binds the designed target sequence GCG-GCG-GCG in the oligonucleotide hairpin CCT-CGC-CGC-CGC-GGG-TTT-TCC-CGC-GCC-CCC GAG G (SEQ ID NO:40) with an affinity of 9nM, as compared to an affinity of 300 mM for an oligonucleotide encoding the GCG-TGG-GCG sequence (as determined by surface plasmon resonance studies). Proteins containing 2 to 12 copies of the C7 finger have been constructed and shown to have specificity for their predicted targets as determined by ELISA (see for example, EXAMPLE 7). Fingers utilized need not be identical and may be mixed and matched to produce proteins which recognized a desired target sequence. These may also be utilized with leucine zippers (*e.g.*, Fos/Jun) to produce proteins with extended sequence recognition.